CLINICAL AND VACCINE IMMUNOLOGY, Apr. 2010, p. 572–581 Vol. 17, No. 4
1556-6811/10/$12.00 doi:10.1128/CVI.00467-09

Protection of Nonhuman Primates against Two Species of Ebola Virus Infection with a Single Complex Adenovirus Vector▽

William D. Pratt,[1]* Danher Wang,[2] Donald K. Nichols,[1] Min Luo,[2] Jan Woraratanadharm,[2] John M. Dye,[1] David H. Holman,[2] and John Y. Dong[2,3]

U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter St., Fort Detrick, Maryland 21702-5011[1]; Division of Biodefense Vaccines, GenPhar, Inc., 600 Seacoast Pkwy., Mount Pleasant, South Carolina 29464-8247[2]; and Department of Microbiology and Immunology, Medical University of South Carolina, 173 Ashley Ave., BSB 201, Charleston, South Carolina 29425[3]

Received 12 November 2009/Returned for modification 18 January 2010/Accepted 10 February 2010

Ebola viruses are highly pathogenic viruses that cause outbreaks of hemorrhagic fever in humans and other primates. To meet the need for a vaccine against the several types of Ebola viruses that cause human diseases, we developed a multivalent vaccine candidate (EBO7) that expresses the glycoproteins of *Zaire ebolavirus* (ZEBOV) and *Sudan ebolavirus* (SEBOV) in a single complex adenovirus-based vector (CAdVax). We evaluated our vaccine in nonhuman primates against the parenteral and aerosol routes of lethal challenge. EBO7 vaccine provided protection against both Ebola viruses by either route of infection. Significantly, protection against SEBOV given as an aerosol challenge, which has not previously been shown, could be achieved with a boosting vaccination. These results demonstrate the feasibility of creating a robust, multivalent Ebola virus vaccine that would be effective in the event of a natural virus outbreak or biological threat.

The filoviruses, Ebola virus (EBOV) and Marburg virus (MARV), cause outbreaks of severe hemorrhagic fever disease in humans, with case-fatality rates that range up to 90%. Among the *Ebolavirus* genus, there are four distinct species: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Reston ebolavirus* (REBOV), and *Cote d'Ivoire ebolavirus* (CIEBOV) (10), with a possible fifth species identified in a recent outbreak in the Bundibugyo region of Western Uganda (34). Of these, ZEBOV and SEBOV are known to cause lethal disease in humans. The persistence of these viruses in nature is not well understood. Sporadic outbreaks due to EBOV have been occurring in Central Africa since the 1970s, but since the mid-1990s, the incidence of outbreaks has increased more than 4-fold (6, 7, 8, 42–45), and EBOV has spread aggressively throughout the great ape sanctuaries of West and Central Africa, decimating wild populations of gorillas and chimpanzees (2). While the filoviruses infect both humans and great apes, due to the high mortality rates of the infection, neither is thought to serve as reservoirs for these viruses but only as accidental hosts (16). Recent findings suggest that African fruit bats may serve as a reservoir host for filoviruses (2, 22); however, little is known about the nature of transmission to humans and nonhuman primates from bats or the likelihood of other reservoir species. In outbreak situations, filoviruses are believed to transmit from person to person mainly through contact with bodily fluids from infected patients. However, recent studies of Ebola outbreaks in wild apes have suggested that there could be other modes of transmission, including aerosol (2, 36). Studies in nonhuman primates have shown that EBOV and MARV can be spread through aerosolized droplets under controlled laboratory conditions (18, 21). So, despite the low incidence of infections globally, the lethality and potential airborne transmission of filoviruses in heavily populated areas makes them a significant biological threat, resulting in their placement on the Centers for Disease Control and Prevention list of Category A Bioterrorism Agents and the Department of Health and Human Services (DHHS) list of select agents and toxins. Concern is further compounded by the potential for these agents to be obtained from the wild (2, 22). In a biological threat scenario, aerosol transmission will likely be the main mode of viral dissemination, and protection against aerosolized EBOV would be of utmost importance (3). However, most previous vaccine candidates have only been evaluated for efficacy against intramuscular or intraperitoneal challenge and not against an aerosol challenge in nonhuman primates.

At present, there are no licensed vaccines or specific antiviral treatments available for EBOV or MARV infections. However, significant progress has been made over the past few years in developing vaccine candidates that can protect nonhuman primates (NHPs) from lethal EBOV and MARV challenges (11, 17, 20, 23, 32, 40, 41). Most of the candidates utilize recombinant vaccine approaches that direct the protective immune response toward the surface glycoprotein (GP) of a single species of EBOV. Importantly, each species of EBOV is antigenically distinct, based on the sequences of the viral GP (10), and therefore, vaccines targeted against the GP of one species of the virus will not provide cross-protection against infection by another (19). Unique among the vaccine candidates is the recombinant complex adenovirus vaccine (CAdVax) system, which provides multivalent protection of NHPs against multiple species of filoviruses (33). The CAdVax vaccine platform is based on a complex, replication-defective adenovirus 5 (Ad5) vector (28–30, 37, 38) that allows for the incorporation of multiple gene inserts into the vector's ge-

* Corresponding author. Mailing address: U.S. Army Medical Research Institute of Infectious Diseases, 1425 Porter St., Fort Detrick, MD 21702-5011. Phone: (301) 619-2405. Fax: (301) 619-2290. E-mail: williamd.pratt@us.army.mil.

▽ Published ahead of print on 24 February 2010.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
10 FEB 2010	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Protection of nonhuman primates against two species of Ebola virus infection with a single complex Adenovirus vector. Clin Vacc Immunol 17:572-581	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Pratt, WD Wang, D Nichols, DK Luo, M Woraratanadharm, J Dye, JM Holman, DH Dong, JY	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	**TR-09-052**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Ebola viruses are highly pathogenic viruses that cause outbreaks of hemorrhagic fever in humans and other primates. To meet the need for a vaccine against the several types of Ebola viruses that cause human diseases, we developed a multivalent vaccine candidate (EBO7) that expresses the glycoproteins of Zaire ebolavirus (ZEBOV) and Sudan ebolavirus (SEBOV) in a single complex adenovirus-based vector (CAdVax). We evaluated our vaccine in nonhuman primates against the parenteral and aerosol routes of lethal challenge. EBO7 vaccine provided protection against both Ebola viruses by either route of infection. Significantly, protection against SEBOV given as an aerosol challenge, which has not previously been shown, could be achieved with a boosting vaccination. These results demonstrate the feasibility of creating a robust, multivalent Ebola virus vaccine that would be effective in the event of a natural virus outbreak or biological threat.

15. SUBJECT TERMS

filovirus, Ebola Zaire, Sudan, multivalent vaccine, parenteral, aerosol, efficacy, laboratory animals, nonhuman primates

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**10**	

nome. Using this design, a bivalent vaccine vector (EBO7) was developed that expresses modified GP gene sequences of SEBOV and ZEBOV. When included in a novel pan-filovirus vaccine formulation, this vaccine was 100% protective in NHPs against two species of EBOV (ZEBOV and SEBOV) and two different strains of MARV (Musoke and Ci67) (33).

In the study presented here, we further tested the protective efficacy of the CAdVax-based EBO7 vaccine in macaques by comparing aerosol to parenteral challenge. Aerosol challenge is potentially even more lethal than parenteral infection, because it induces hemorrhagic pneumonia. This is particularly true of SEBOV aerosol challenge, against which protection has not previously been demonstrated. In our studies, we have found that for either route of infection, the vaccine-induced bivalent anti-EBOV responses were protective against lethal challenge with either SEBOV or ZEBOV. This is the first report of a vaccine that is capable of protecting against aerosol SEBOV challenge. In addition, we found that EBO7 was also capable of protecting macaques with preexisting immunity to adenovirus against ZEBOV challenge. These results provide further insight into the feasibility of developing a fully protective multivalent EBOV vaccine using the CAdVax vaccine platform.

MATERIALS AND METHODS

Viruses and CAdVax vaccine vectors. The challenge viruses, ZEBOV (Kikwit strain) (15) and SEBOV (Boniface strain) (13), were specific challenge stocks that were developed at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) for NHP studies. The CAdVax-based EBO7 vector contains modified GP gene sequences from both the Boniface strain of SEBOV and the Kikwit strain of ZEBOV (GenBank accession numbers U28134 and U28077, respectively). These genes were modified to delete the RNA editing signal responsible for initiating a secreted, nonstructural form of GP (38). Both genes were amplified by PCR and then subcloned into pLAd (SEBOV GP) or pRAd (ZEBOV GP) plasmid shuttle vectors (28–30, 37, 38). Using these shuttle vectors, the vaccine was constructed as previously described (28–30, 37, 38). The genomic DNA from the final vaccine vector was confirmed by restriction digest mapping, PCR, and sequence analyses. The M8 vector expresses the GP antigens from the Ci67 strain (MARV-Ci67) and Ravn strain (MARV-Ravn) of MARV and was described previously (33). In some experiments, M8 was included in the CAdVax-EBO7 vaccine to test for any possible immune interference that might occur as a consequence of simultaneous vaccination with EBO7. The control vaccine vector, HC4, is a CAdVax-based hepatitis C vaccine vector.

Vaccine production, analysis, and titration. The CAdVax vector genome is devoid of E1, E3, and most of E4 (with the exception of ORF6) (30). However, this vector is still capable of efficient replication in the standard human embryonic kidney 293 (HEK293) cell line, which provides E1 in *trans*. Each CAdVax vector was propagated in HEK293 cells obtained from the American Type Culture Collection (Manassas, VA) using standard procedures (28–30). The vector was processed through at least three rounds of single-plaque selection, followed by genome screening of vector clones for the correct transgene inserts using restriction mapping digestion, PCR, and DNA sequencing analyses. This ensured that no genetic deletions or rearrangements had occurred during the vaccine propagation steps. These steps were carried out for EBO7, as well as for M8 and HC4. Bench-scale lots of the final vaccine vectors were purified by ultracentrifugation in cesium chloride gradients as previously described (28) and stored frozen in 1-ml aliquots in liquid nitrogen.

EBO7, M8, and HC4 were titrated on HEK293 cells infected with serial dilutions of each respective vector in 12-well plates according to a standard adenovirus plaque assay (9). Seven days after infection, plaques were counted and the resulting titers were scored as PFU per ml. The antigen gene sequences were confirmed again with restriction mapping digestion. Protein expression from each vaccine component was confirmed by Western blotting, immunofluorescence assay, and immunogenicity in mice as previously described (38, 39).

Animal studies. Twenty-five (3 to 5 kg) cynomolgus macaques (*Macaca fascicularis*) and 18 rhesus macaques (*Macaca mulatta*) (3 to 6 kg) were used for these studies. In all animal challenge studies, the target dose of virus was 1,000 PFU. Back-titration values are given in the respective tables (Tables 1 to 3). For the parenteral challenge studies, cynomolgus macaques were vaccinated intramuscularly (i.m.) on day zero with a 1:1 mixture of 1×10^{10} PFU each of EBO7 and M8 (total 2×10^{10} PFU). Control animals received an i.m. injection of 2×10^{10} PFU of HC4. The same animals were given boosting vaccinations of the same dose and route on either day 65 or day 120, depending on the experiment (Fig. 1). Six weeks after the boosting vaccinations, the macaques were anesthetized by i.m. injection of Telazol (2 to 6 mg/kg of body weight) and then inoculated i.m. with SEBOV or ZEBOV challenge stock. All animals were closely monitored for 28 days after the challenge inoculations. Where stated below, some animals were back-challenged with an i.m. injection of ZEBOV 10 weeks after the initial SEBOV challenge.

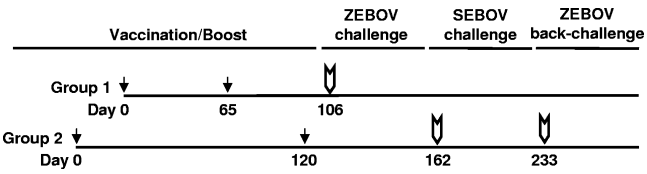

FIG. 1. Vaccination and challenge schedule. Cynomolgus macaques were divided into two groups of five per group, and each group was vaccinated on days 0 and 65 (group 1) or days 0 and 120 (group 2) with CAdVax-EBO7 or a control CAdVax vector. Group 1 was challenged with 1,000 PFU of ZEBOV on day 106, and group 2 was challenged with 1,000 PFU of SEBOV on day 162 (both groups were challenged 6 weeks post-boosting vaccination). After a 10-week recovery period, group 2 was subsequently back-challenged with 1,000 PFU of ZEBOV on day 233. Filled arrows, vaccination; open arrows, virus challenge.

For the initial aerosol infection experiments, cynomolgus macaques were vaccinated by i.m. injection of 1×10^{10} PFU of EBO7 or 1×10^{10} PFU of HC4. Twenty-eight days after vaccination, animals were anesthetized and exposed to a target dose of 1,000 PFU of either aerosolized ZEBOV or aerosolized SEBOV. Aerosolized EBOV was generated with a Collison nebulizer within a Plexiglas chamber contained within a class III biological safety cabinet situated in the animal biosafety level 4 laboratory. For the follow-on aerosol experiment with SEBOV, cynomolgus macaques were vaccinated by i.m. injection of 1×10^{10} PFU of EBO7 or 1×10^{10} PFU of HC4 and given a boosting vaccination with the same vector dose 71 days later. Twenty-eight days after the boosting vaccination, animals were anesthetized and exposed to a target dose of 1,000 PFU of aerosolized SEBOV. All animals were monitored closely for 28 days after challenge.

For the anti-Ad5 immunity study, three rhesus macaques were vaccinated by i.m. injection at weeks 0, 16, and 44 with 1×10^6 PFU of wild-type adenovirus 5. Rhesus macaques were selected for this experiment due to their availability. Use of these animals saved considerable expense by obviating the need to obtain additional animals. Rhesus and cynomolgus macaques are very similar in size and weight and experience the same course and pathology of disease after filovirus infection. The animals were maintained for a total of 52 weeks, with serum samples periodically taken for analysis by enzyme-linked immunosorbent assay (ELISA) for anti-Ad5 reactivity.

For the antivector immunity and challenge studies, nine rhesus macaques were vaccinated by i.m. injection at weeks 0 and 8 with 1×10^9 PFU of a CAdVax-based dengue virus vaccine (previously described in reference 27). These rhesus macaques were chosen primarily because of their preexisting immunity status as a result of separate immunogenicity studies of the dengue vaccine. The animals were maintained for 52 weeks, with serum samples taken at periodic intervals for evaluation of antivector antibody titers. At week 52, the macaques were separated into three groups of three animals each and vaccinated by i.m. injection with a low dose (1×10^8 PFU), medium dose (1×10^9 PFU), or high dose (1×10^{10} PFU) of EBO7. A group of three rhesus macaques which had not previously received the CAdVax-based dengue vaccine were added to the study to serve as naïve controls and were vaccinated with the medium dose of EBO7 at week 56. Blood samples were collected from all animals on a biweekly basis through 12 weeks postvaccination for evaluation of anti-EBOV immune responses. At 1 week before aerosol challenge (approximately week 90), all groups received a boosting vaccination with their respective doses of EBO7. Three naïve macaques were vaccinated at this time with HC4 and served as vector-only controls. On week 91, all groups were anesthetized and exposed to 1,000 PFU of aerosolized ZEBOV, as described above. All animals were monitored closely for 28 days after challenge.

Due to ethical considerations stemming from the near universal lethality of infection and as is standard practice for nonhuman primate studies with filoviruses, historical controls were sometimes used to limit the number of animals required (12, 41). All challenge studies were conducted under maximum containment in an animal biosafety level 4 facility at USAMRIID and were approved by the USAMRIID Institutional Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (24a). The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Hematology, blood biochemistry, and humoral immune responses. Phlebotomy was performed on the femoral vein using a venous blood collection system (Becton Dickinson, Franklin, NJ). Viremia was assayed by traditional plaque assay (24). Hematological values of blood samples collected in tubes containing EDTA were determined using a hematologic analyzer (Coulter Electronics, Hialeah, FL). Liver-associated enzymes were measured using a Piccolo point-of-care blood analyzer (Abaxis, Sunnyvale, CA). To determine the specific antibody titers against SEBOV and ZEBOV, sera were collected from vaccinated and control animals periodically before and after vaccination. Viruses were propagated *in vitro* in Vero E6 cells, purified by sucrose gradient, and inactivated by irradiation. Inactivated SEBOV or ZEBOV preparations were then used to coat polyvinyl chloride (PVC) ELISA plates (Dynatech Laboratories, Chantilly, VA) with 50 μl per well of each virus diluted to a concentration of 1 mg/ml total protein. The coated plates were incubated overnight at 4°C, and the assay was carried out as previously described (33). Antibody titers were defined as the reciprocal of the highest dilution giving a net optical density value of >0.2.

The antivector and anti-ZEBOV ELISAs whose results are shown in Fig. 3 and 4 were conducted as described previously (38). For these studies, CAdVax-VEE vector (a CAdVax-based vaccine expressing an irrelevant antigen) served as the CAdVax vector target for the antivector ELISAs, and the cell culture supernatants from BS-C-1 cells transduced with the CAdVax-EBO3 vector served as the ELISA target for the anti-ZEBOV ELISAs. The CAdVax-EBO3 vector expresses the unmodified ZEBOV GP gene and produces a secreted form, by way of RNA editing (31, 35), into the cell culture supernatant; these secreted GP antigens were collected into serum-free medium to reduce potential background from serum proteins.

Postmortem examination. For all of the animal studies described above, the body of each monkey that succumbed or was euthanized due to the severity of clinical disease was submitted for a gross necropsy under biosafety level 4 containment. Gross necropsies were also performed on the animals that survived the aerosolized SEBOV challenge experiments.

During the gross necropsies, samples of the following organs were collected from each animal and fixed in 10% buffered formalin for histology: liver, spleen, kidney, adrenal gland, gonad, axillary lymph node, inguinal lymph node, and mesenteric lymph node. In addition, for those monkeys in the aerosol challenge studies that succumbed and those that were euthanized at the end of the aerosolized SEBOV studies, histologic samples of tongue, mandibular lymph node, larynx, trachea, lungs, and mediastinal lymph nodes were also collected. The set of formalin-fixed tissue samples from each monkey was held for a minimum of 21 days under biosafety level 4 containment and then was decontaminated and transferred to the USAMRIID histopathology laboratory. All tissue samples were then trimmed, routinely processed, and embedded in paraffin. Sections of the paraffin-embedded tissues 5 μm thick were cut for histology. The histology slides were deparaffinized, stained with hematoxylin and eosin (H&E), and placed under coverslips.

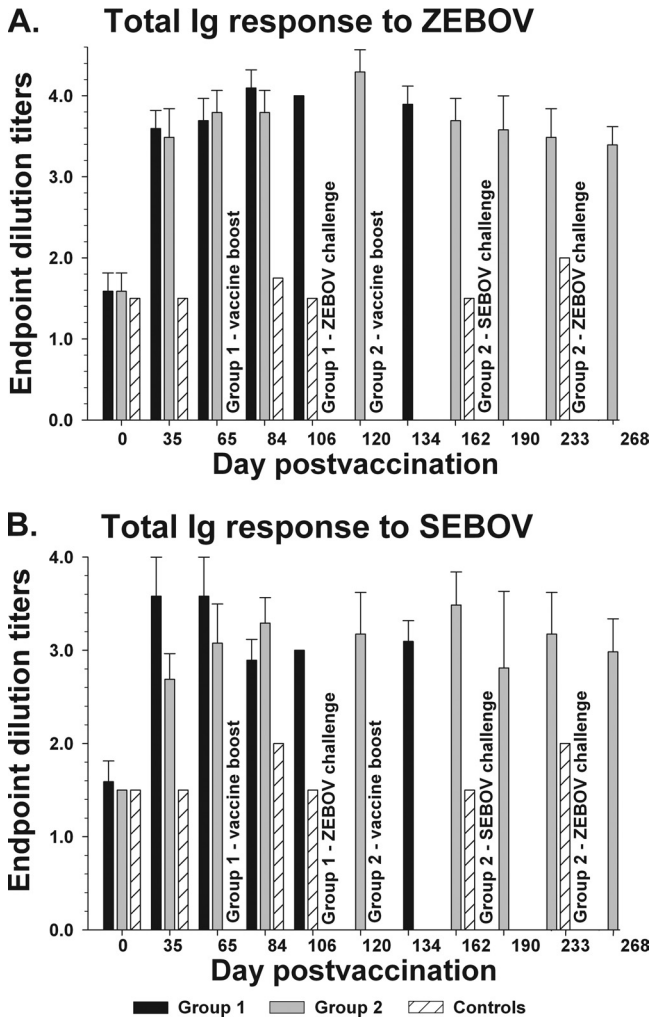

FIG. 2. Humoral immune responses to ZEBOV and SEBOV before and after challenges. Geometric mean titers (plus standard deviations) of total immunoglobulin (Ig) in response to ZEBOV (A) and SEBOV (B) were measured by ELISA using inactivated filovirus preparations as immune targets. Group 1 animals were challenged with ZEBOV only. Group 2 animals were challenged with SEBOV and then back-challenged with ZEBOV. The control NHPs ($n = 3$) were evaluated on day zero before challenge.

RESULTS

Vaccination of cynomolgus macaques with EBO7 induces bivalent antibody responses. In order to evaluate vaccine-mediated anti-EBOV immune responses *in vivo*, we vaccinated five cynomolgus macaques by i.m. injection with an equal mixture of 1×10^{10} PFU each of EBO7 and M8 on day 0 and day 65 (Fig. 1, group 1). Using indirect ELISA antibody analyses, we found that all vaccinated macaques generated high antibody titers against both ZEBOV and SEBOV after vaccination (Fig. 2, group 1). These titers increased to 3.5 $\log_{10}$ to 4.0 $\log_{10}$ within 5 weeks of the primary vaccination and remained relatively constant after the boosting vaccination at day 65 and after ZEBOV challenge (day 106). Anti-SEBOV responses were similar to those against ZEBOV, indicating that EBO7 elicited high levels of balanced bivalent humoral immune responses in vaccinated macaques. Furthermore, the inclusion of M8 with EBO7 did not appear to have any negative impact on the humoral immunogenicity of EBO7 compared to the response of macaques vaccinated with just EBO7. This second group of cynomolgus macaques, group 2, was vaccinated with the same dose of EBO7 vaccine only but, due to scheduling constraints in the biosafety level 4 suite, had to be boosted on day 120 rather than day 65. This change in vaccination schedule did not appear to have any significant effect on the induction of humoral immune responses, as the anti-ZEBOV and anti-SEBOV antibody titers in the group 2 macaques were similar to the levels seen in the group 1 macaques (Fig. 2).

TABLE 1. CAdVax-EBO7 protects cynomolgus macaques against both ZEBOV and SEBOV challenge

Group[a]	Vaccine	Initial challenge[b]		Back-challenge[b]		Clinical findings[c]	Outcome
		Virus	S/T (%)	Virus	S/T (%)		
Group 1	EBO7/M8	ZEBOV	5/5 (100)			No clinical signs	All survived
Control A	Mock	ZEBOV	0/1 (0)			F, D, A, moderate P, V (d3, 4.3; d5, 6.8), T, LE ↑ ↑ ↑	Succumbed on day 6
Group 2	EBO7/M8	SEBOV	5/5 (100)			No clinical signs	All survived
Control B	Mock	SEBOV	0/1 (0)			D, A, mild P, V (d7, 6.1; d10, 6.7), T, LE ↑ ↑	Succumbed on day 10
Group 2	EBO7/M8			ZEBOV	5/5 (100)	No clinical signs	All survived
Control C	Mock			ZEBOV	0/1 (0)	F, D, A, mild P, V (d5, 3.7; d7, 5.4), T, LE ↑ ↑ ↑	Succumbed on day 9

[a] Group 1 macaques were vaccinated on days 0 and 65 with 1×10^{10} PFU each of EBO7 and M8 and then challenged on day 106 with ZEBOV. Group 2 macaques were vaccinated on days 0 and 120 with 1×10^{10} PFU each of EBO7 and M8 and then challenged on day 162 with SEBOV. Control macaques (A, B, and C) were vaccinated with 2×10^{10} PFU of HC4.

[b] Based on back-titration, group 1 and control A were challenged with 500 PFU of ZEBOV, group 2 and control B were challenged with 800 PFU of SEBOV, and group 2 was back-challenged with 1,100 PFU of ZEBOV along with control C. In addition to these controls, historical control macaques ($n = 20$ for ZEBOV, and $n = 10$ for SEBOV) were used to limit the number of controls. S/T, number of survivors/total number challenged.

[c] Clinical findings: fever (F) was defined as a rectal temperature increase of more than 2°C over baseline; depression (D) and anorexia (A) were assessed subjectively; petechia (P) was defined as mild (barely visible), moderate (visible over focal areas) or widespread; viremia (V) was defined as detectable virus in the serum, with the day (d) of detection and $\log_{10}$ value in PFU/ml shown in parentheses; thrombocytopenia (T) was defined as a ≥35% decrease in platelets; and elevated levels of liver-associated enzymes (LE) were defined as a 2- to 3-fold increase (↑), a 4- to 5-fold increase (↑ ↑), or a more-than-5-fold increase (↑ ↑ ↑) in serum aspartate aminotransferase and alanine aminotransferase over baseline.

Vaccination of cynomolgus macaques with EBO7 provides bivalent protection against both ZEBOV and SEBOV. To test the ability of EBO7 to protect against both types of EBOV, group 1 macaques were challenged with an i.m. injection of 500 PFU ZEBOV at 6 weeks after the boosting vaccination (Table 1, group 1). All five EBO7-vaccinated macaques survived the lethal challenge with no signs of disease, whereas the control macaque (control A) developed typical signs of disease (fever, depression, anorexia, viremia, thrombocytopenia, petechia, and elevated levels of liver-associated enzymes in the serum) and succumbed on day 6. To test the bivalent efficacy of EBO7, group 2 macaques were challenged with an i.m. injection of 800 PFU of SEBOV at 6 weeks after the boosting vaccination (Table 1, group 2). All EBO7-vaccinated macaques survived the lethal challenge with no signs of disease, while the control macaque (control B) succumbed on day 10. As an additional test, the group 2 macaques were allowed to recover for approximately 10 weeks and then were back-challenged with an i.m. injection of 1,100 PFU ZEBOV on day 233. As before, all five vaccinated macaques survived the second lethal challenge with a different type of virus without developing any signs of disease, while the control macaque (control C) succumbed on day 10 with typical symptoms and signs of the infection (Table 1). Postmortem examination confirmed that each of the three control animals in the above-described experiments succumbed due to EBOV infection and that there were no other confounding factors or underlying diseases.

Vaccination of cynomolgus macaques with EBO7 provides protection against aerosolized EBOV challenge. The results of our previous experiments in cynomolgus macaques (33), as well as the data in Table 1, suggest that the primary vaccinations had induced maximal levels of immune responses and that boosting vaccinations with CAdVax vaccines may not have been required. As shown in Fig. 2 (for both groups 1 and 2), anti-ZEBOV and anti-SEBOV titers rose to peak levels within each group by day 35, and boost vaccination did not further increase the level of antibody responses. This interpretation is also supported by the fact that the immune responses did not increase after the challenges with live virus. These data, and results from other vaccine platforms where a single-dose vaccination protected against challenge by a single Ebola virus species (12, 19, 32), compelled us to test whether a single dose of EBO7 could provide bivalent protection against lethal aerosolized EBOV challenges.

In the first study, three cynomolgus macaques were vaccinated by i.m. injection with a single dose of 1×10^{10} PFU of EBO7 on day zero and were challenged 28 days later with 900 to 1,000 PFU of aerosolized ZEBOV. All three EBO7-vaccinated macaques survived the aerosol challenge with ZEBOV without any signs of disease, demonstrating that a single dose of the CAdVax-based EBO7 vaccine was sufficient to provide complete protection against an aerosol ZEBOV challenge (Table 2, study 1). As expected, the aerosol control animal (control 1) succumbed on day 7, with signs of disease similar to those seen in control macaques challenged by the parenteral route and in the historical controls (data not shown); the postmortem examination results confirmed that this monkey succumbed due to EBOV infection and that there were no other confounding factors.

In the second study, three cynomolgus macaques were vaccinated by i.m. injection with a single dose of 1×10^{10} PFU of EBO7 on day zero and were challenged 28 days later with 100 to 500 PFU of aerosolized SEBOV (Table 2, study 2). Two of three vaccinated macaques survived the aerosol challenge and showed no clinical signs of disease. However, one of the EBO7-vaccinated macaques succumbed on day 10. Interestingly, this macaque had few clinical signs of disease, primarily anorexia and profound depression, and the most prominent pathological features from this animal were present in the thoracic organs. Multifocal fibrinosuppurative necrotizing pneumonia and acute pleuritis were present in the lungs, and the mediastinal lymph nodes contained multiple foci of necrosis and acute inflammation accompanied by edema and inflammation of the surrounding mediastinal connective tissue. The immunohistochemistry results revealed viral antigen located within monocytes, macrophages, and fibroblasts in and around areas of inflammation in the lungs and mediastinal lymph nodes; no viral antigens were detected in any other

TABLE 2. EBO7 protects cynomolgus macaques against aerosolized EBOV challenge

Study	Group[a]	Vaccine	No. of doses	ELISA titer[b]	Aerosol challenge[c]		Clinical findings[d]	Outcome
					Virus	S/T (%)		
1	Group 1	EBO7	1	Z, 3.2; S, 3.7	ZEBOV	3/3 (100%)	No clinical signs	All survived
	Control A	Mock	1		ZEBOV	0/1 (0)	F, D, A, moderate P, V (d5, 5.6), T, LE ↑ ↑ ↑	Succumbed on day 6
	Group A historical controls				SEBOV	0/3 (0)	D, A, no P, T, dyspnea, no blood work	Succumbed on day 9, 9, or 12
2	Group 2	EBO7	1	Z, 3.5; S, 3.5*	SEBOV	2/3 (67)	One with clinical signs: D, A, no viremia detected	One succumbed on day 9
	Control B	Mock	1		SEBOV	0/1 (0)	D, A, widespread P, V, LE ↑ ↑	Succumbed on day 8
	Group B historical controls				SEBOV	0/3 (0)	D, A, widespread P, V, LE ↑ ↑– ↑ ↑ ↑	Succumbed on day 7, 8, or 9
3	Group 3	EBO7	2	Z, 3.8; S, 4.7*	SEBOV	3/3 (100)	One with clinical signs: D, A, no viremia detected	All survived
	Control C	Mock	2		SEBOV	0/1 (0)	D, A, widespread P, V (d3, 2.6; d5, 6.1; d7, 7.3), T, LE ↑ ↑ ↑	Succumbed on day 7

[a] Group 1 and 2 macaques were vaccinated with 1×10^{10} PFU of EBO7 28 days before aerosol challenge. Group 3 macaques were vaccinated on days 0 and 71 with 1×10^{10} PFU of EBO7 and then challenged 28 days after the boosting vaccination. Control animals were vaccinated with 1×10^{10} PFU of HC4. In addition to these controls, historical control macaques ($n = 7$ for ZEBOV, and $n = 6$ for SEBOV) were used to limit the number of controls.

[b] Geometric mean titers ($\log_{10}$) of total serum immunoglobulin as measured by ELISA using inactivated ZEBOV (Z) or SEBOV (S) preparations as targets. *, SEBOV-specific titers were significantly higher in macaques receiving two doses of EBO7 (group 3) than in macaques receiving a single dose (group 2) ($P < 0.02$).

[c] Based on back-titration, group 1 and control A were aerosol challenged with 900 to 1,000 PFU of ZEBOV; group A historical controls were aerosol challenged with approximately 20 PFU of SEBOV. Group B historical controls, groups 2 and 3, and controls B and C were aerosol challenged with 100 to 500 PFU of SEBOV. S/T, number of survivors/total number challenged.

[d] Fever (F) was defined as a rectal temperature increase of more than 2°C over baseline; depression (D) and anorexia (A) were assessed subjectively; petechia (P) was defined as mild (barely visible), moderate (visible over focal areas), or widespread; viremia (V) was defined as detectable virus in the serum, with the day (d) of detection and $\log_{10}$ value in PFU/ml shown in parentheses; thrombocytopenia (T) was defined as a ≥35% decrease in platelets; and elevated levels of liver-associated enzymes (LE) were defined as a 2- to 3-fold increase (↑), a 4- to 5-fold increase (↑ ↑), or a more-than-5-fold increase (↑ ↑ ↑) in serum aspartate aminotransferase and alanine aminotransferase over baseline.

tissues from this monkey. These histologic lesions and immunohistochemistry results indicated that the EBOV infection in this macaque was restricted to the lungs and their draining lymph nodes. There were no lesions and no viremia or viral antigen findings indicative of a systemic viral infection in typical target organs, such as liver, spleen, adrenals, and reproductive tract. These data support our conclusion that the death of this monkey was due to a restricted respiratory viral infection that has not been seen previously in parenteral challenges. Interestingly, the serum virus-specific ELISA titers of this macaque were not different than those of the two survivors. The control macaque in study 2 (control B) had pulmonary lesions that were similar to but much less severe than those seen in the vaccinated macaque that succumbed to challenge. In contrast to the vaccinated animal, this control monkey also had profound viremia and EBOV-induced lesions in the liver, spleen, reproductive tract, adrenals, and multiple lymph nodes that were typical of those that occur in macaques with disseminated EBOV infections; immunohistochemistry confirmed the presence of viral antigens in these lesions and in those located in the respiratory tract. The historical controls (Table 2, group A) had mild to moderate pneumonia and pleuritis, as well as lesions characteristic of disseminated EBOV infection. After study 2, a separate group of historical controls (Table 2, group B) was acquired, and these macaques showed pulmonary lesions similar to those seen in control B. In all of the control macaques ($n = 8$), the lesions present in the liver, spleen, adrenals, intestinal tract, and reproductive tract were consistent with those that occur in macaques that have been experimentally infected with EBOV by challenge routes other than aerosol. There were no signs of other underlying diseases in any of the control subjects. The two vaccinated monkeys that survived were euthanized on day 28 postchallenge; postmortem examination of these monkeys did not reveal any significant virus-induced lesions, and immunohistochemistry did not detect viral antigens in any tissues, indicating complete protection by the vaccine.

The death of one of the three animals vaccinated with a single dose against aerosol SEBOV challenge prompted us to repeat the aerosol SEBOV challenge study using a two-dose regimen (Table 2, study 3). Cynomolgus macaques were vaccinated on day 0 with 1×10^{10} PFU of EBO7, followed by a boosting vaccination on day 71 and challenge on day 99. All of the vaccinated animals were protected from lethal aerosol challenge, while the control animal succumbed on day 7. Similar to what was seen in the control animals described above, this control macaque had moderate to marked multifocal pneumonia and pleuritis in addition to the profound viremia and lesions that are typical of disseminated EBOV infection. One of the vaccinated macaques did show anorexia and mild depression for 3 days but had no viremia or other clinical signs of disease. The other two vaccinated macaques showed no clinical signs of disease. At 28 days after challenge, postmortem examination of the vaccinated macaque that showed clinical signs revealed that it had multiple foci of moderate chronic bronchopneumonia and fibrous pleural adhesions. These types of chronic lesions are not known to be associated with typical Ebola virus infection in experimental infections, and there was no evidence of active viral infection, and no viral antigens were detected by immunohistochemistry in these lesions. The foci

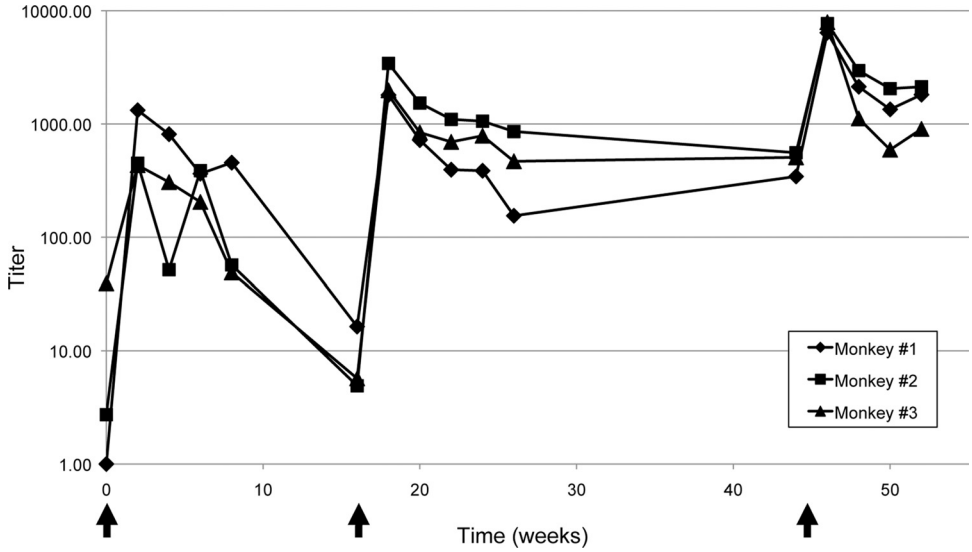

FIG. 3. Ad5 antibody responses after repeated exposure. Three monkeys were injected i.m. three times (arrows) with 10^6 PFU of wild-type Ad5. Animal serum samples were used to measure Ad5-reactive antibodies by ELISA. After each injection, anti-Ad5 antibody titers increased rapidly and then began to drop, but they remained well above the preinjection level, indicating a sustained response.

were likely to be the sequelae of EBOV infection confined by the immune responses. At necropsy, one of the remaining vaccinated macaques also had minor fibrous thoracic adhesions, while the third had no lesions. There were no signs of infection found in other vital organs in any of the vaccinated macaques. These results show that the multivalent EBO7 vaccine can protect macaques against lethal challenge with both ZEBOV and SEBOV by the aerosol route. However, two vaccine doses were required to protect against lethality by aerosol SEBOV and this route of SEBOV infection appears to be more difficult to fully protect against due to its propensity to cause pneumonia and pleuritis.

Vaccination provides complete protection against aerosol ZEBOV challenge, even in the presence of preexisting vector immunity. The view that preexisting immunity to adenovirus (Ad) may impact Ad vector use in humans is based on the observation that a significant proportion of the world's population has neutralizing antibodies to Ad subtypes such as serotype 5 (Ad5). While natural Ad infection may be prevented in these people by neutralization of the few transmitted viral particles, we hypothesized that a high dose of Ad vector, as is delivered locally in a vaccination ($\geq 1 \times 10^8$ particles), would overcome any memory immunity previously established. Two experiments were conducted to investigate this hypothesis.

To demonstrate an Ad5 immune response, we vaccinated rhesus macaques with recombinant Ad5 virus, collected serum samples periodically over the course of 1 year, and assayed for anti-Ad5 antibodies. Three animals each displayed classical primary and secondary immune response patterns (Fig. 3). After the first vaccination, the monkeys mounted a somewhat slow and weak humoral immune response against Ad5. However, after the second and third inoculations, all three animals showed a rapid and robust increase in anti-Ad5 antibodies, indicating the existence of a memory immune response. These periods of elevated immunity likely mimic an acute Ad5 infection, and a CAdVax vaccination at these points would be expected to be less effective due to the higher levels of anti-Ad5 antibody titers. However, by 8 to 10 weeks postvaccination, the antibody titers fell by between 0.5 to 1.0 log and appeared to remain sustained at that level. This sustained lower-level immune response is a good model to mimic preexisting immunity in humans that have been exposed to Ad5 infection. Based on the results of this study, we designed a second experiment testing the feasibility of vaccinating Ad5-immune macaques with EBO7.

To generate Ad5 immunity, we vaccinated 9 rhesus macaques with 1×10^9 PFU of an unrelated CAdVax vector at weeks 0 and 8. Serum samples were collected and used to measure Ad5-reactive antibodies over the course of 1 year (Fig. 4, black squares). There are two reasons that we used a nonreplicating vector instead of wild-type Ad5 to generate preexisting immunity. First, during a natural infection, wild-type Ad will replicate from a few particles to a titer high enough to induce protective immune responses. However, there are significant differences in the levels of Ad5 replication in different individuals, necessitating a large number of animals for the study. In contrast, using nonreplicating vectors, the final titer of Ad5 in each animal is controlled by the injection dose, such as 10^9 PFU, to create a similar level of immune response in genetically diverse NHPs. The second reason is the limitations and ethical considerations of using NHPs. By using an unrelated vaccine vector, we could utilize the same group of NHPs for two sets of experiments in which the first vaccination in the first set served to induce antivector immunity for the second set.

After the primary Ad5 vaccination, the macaques were housed for 52 weeks (~1 year) to allow the acute anti-Ad5 immune response to fall to memory immunity levels, which we believe closely mimic preexisting immunity in humans. The animals were then separated into three groups of three and vaccinated with 10^8 PFU (low dose), 10^9 PFU (medium dose), or 10^{10} PFU (high dose) of EBO7. As a control for immuno-

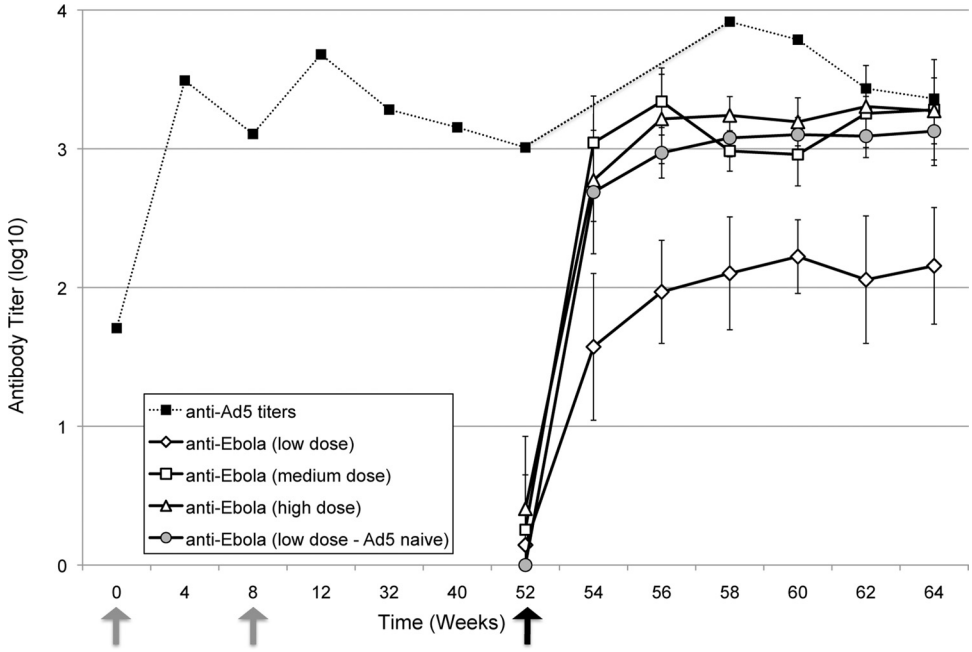

FIG. 4. Increasing the CAdVax vaccine dose is sufficient to overcome preexisting Ad5 immunity. Rhesus macaques were vaccinated twice at weeks 0 and 8 with an unrelated CAdVax vector to induce Ad5 immunity (gray arrows). At week 52 (black arrow), the animals were separated into three groups ($n = 3$) and vaccinated with 1×10^8 PFU (low dose, open diamonds), 1×10^9 PFU (medium dose, open squares), or 1×10^{10} PFU (high dose, open triangles) of EBO7. As a control, a group of Ad5-naïve animals was vaccinated with a low dose of EBO7 (shaded circles). Antibody titers were determined for Ad5 (black squares) and ZEBOV (open or shaded symbols) GP. Error bars show standard deviations.

genicity, a fourth group, comprised of Ad5-naïve animals, received a low dose (10^8 PFU) of EBO7 vaccine. As shown by the results in Fig. 4, Ad5-immune macaques vaccinated with either the medium or high dose mounted strong anti-ZEBOV humoral responses (Fig. 4, open squares and triangles) that were comparable to those in Ad5-naïve macaques (Fig. 4, shaded circles), while Ad5-immune macaques vaccinated with the low dose had reduced anti-ZEBOV titers in comparison, suggesting that a portion of the vaccine was indeed neutralized by the preexisting immunity. These collective data demonstrate that increasing the dose of vaccine by one log, from 10^8 to 10^9 PFU, was sufficient to compensate for the portion of the vaccine neutralized by preexisting immunity or to overcome the preexisting Ad5 immunity. To determine if ZEBOV-specific immune responses in EBO7-vaccinated Ad5-immune macaques were protective, we boosted and then challenged these macaques with ZEBOV. As shown in Table 3, all Ad5-immune NHPs, whether vaccinated and boosted with 10^8, 10^9, or 10^{10} PFU of EBO7, were fully protected against aerosol ZEBOV challenge. Thus, even in those Ad5-immune NHPs receiving the low-dose EBO7, where this preexisting immunity reduced the initial anti-ZEBOV antibody titers, the vaccine was able to elicit a protective response against aerosolized ZEBOV challenge with a two-dose vaccination. In contrast, the control animals that received control CAdVax vaccines all succumbed to infection after the aerosolized ZEBOV challenge; this was confirmed by the necropsy and histology results. In addition, this study was designed to test the protective immune response of a boosting vaccination in a scenario analogous to a military deployment or outbreak situation where personnel are mobilized to an area of possible exposure. We found that all the Ad5-immune groups of macaques vaccinated at the different doses responded with essentially equivalent high serum ELISA titers within 7 days of the boost and were protected against lethal challenge. This opens up the possibility of using a boost vaccination to achieve quick and complete protection in an emergency situation.

DISCUSSION

Considering the potential of EBOV as a biological threat, an effective, safe, and rapidly acting, broadly protective vaccine is of critical importance. In this study, we focused our efforts on a number of issues proven to be challenging in the development of EBOV vaccines. First, each of the five known EBOV species is antigenically distinct. Vaccines against one species of EBOV have been shown not to cross-protect against a different EBOV species (19), and therefore, a monovalent vaccination strategy may be ineffective if the outbreak strain of virus differs from the vaccine EBOV strain. Ideally, a broadly protective vaccine that protects against multiple deadly species of EBOV would be highly desirable, especially in the early phases of an outbreak or biological incident—a period of time before the identification of the causative species of virus. As an approach to developing a multivalent EBOV vaccine, we expressed modified GP antigens from the two major species of EBOV that cause deadly disease in humans, ZEBOV and SEBOV, using a single CAdVax vaccine component, EBO7. In this study, we demonstrated that a single vaccine, EBO7, protected cynomolgus macaques against both ZEBOV and SEBOV.

Animals vaccinated with EBO7 responded with a rapid increase in antibody titer against both ZEBOV and SEBOV

TABLE 3. EBO7 protects rhesus macaques with preexisting immunity to CAdVax against aerosolized ZEBOV challenge

Group	Ad5 status[a]	Vaccine dose (PFU)[b]		ZEBOV ELISA titer[c]			Clinical findings[d]	Outcome [S/T[e] (%) or day of death]
		Prime	Boost	Prime	Boost	Day 14		
Group 1	Immune	10^{10}, EBO7	10^{10}, EBO7	2.6	3.8	5.0	No clinical signs	3/3 (100)
Group 2	Immune	10^{9}, EBO7	10^{9}, EBO7	2.2	4.0	4.8	No clinical signs	3/3 (100)
Group 3	Immune	10^{8}, EBO7	10^{8}, EBO7	1.8	3.0	4.5	No clinical signs	3/3 (100)
Control A	Naïve	5×10^{9}, HC4					D, A, widespread P, V (d7, 3.9), T, LE ↑	9
Control B	Naïve	5×10^{9}, HC4					D, A, widespread P, V (d3, 2.6; d5, 4.9; d7, 5.5), T, LE ↑ ↑ ↑	8
Control C	Naïve	5×10^{9}, HC4					F, D, A, moderate P, V (d5, 3.3; d7, 6.3), T, LE ↑ ↑ ↑	7

[a] Immune, nine rhesus macaques were vaccinated with two doses (10^9 PFU) of an unrelated CAdVax vaccine approximately 1 year before the primary vaccination with EBO7.

[b] The Ad5-immune macaques were divided into three groups and were vaccinated with EBO7. At approximately 34 weeks after the primary vaccination, macaques in groups 1 to 3 received a boosting vaccination of EBO7. At that time, control animals were vaccinated with 5×10^9 PFU of HC4. Seven days later, macaques were aerosol challenged with 800 to 1,200 PFU ZEBOV, based on back-titration.

[c] Geometric mean titers ($\log_{10}$) of total serum immunoglobulin collected at various time points as measured by ELISA using inactivated ZEBOV preparations as targets. Time points: prime is at 34 weeks after the primary vaccination, on the day the macaques received a boosting vaccination of EBO7; boost is on the day of aerosol challenge; and day 14 is 14 days after challenge.

[d] Fever (F) was defined as a rectal temperature increase of more than 2°C over baseline; depression (D) and anorexia (A) were assessed subjectively; petechia (P) was defined as mild (barely visible), moderate (visible over focal areas), or widespread; viremia (V) was defined as detectable virus in the serum, with the day (d) and $\log_{10}$ value in PFU/ml shown in parentheses; thrombocytopenia (T) was defined as a ≥35% decrease in platelets; and elevated levels of liver-associated enzymes (LE) were defined as a 2- to 3-fold increase (↑), a 4- to 5-fold increase (↑ ↑), or a more-than-5-fold increase (↑ ↑ ↑) in serum aspartate aminotransferase and alanine aminotransferase over baseline.

[e] S/T, number of survivors/total number challenged.

antigens. These titers reached maximal levels by 5 weeks and remained maximal until week 12 or longer. Animals receiving booster vaccinations at week 9 or 17 showed no further elevation in antibody titers, suggesting that a single dose of EBO7 induced a strong, long-lived immune response. The lack of an observed effect of the boosting vaccination on total antibody titers might be accounted for by the high titer of antibodies induced by the primary vaccination, which may have already reached maximal biological levels. This is consistent with the observation that vaccinated animals that survived challenge with ZEBOV or SEBOV also showed no further increase in antibody titers in response to the viral antigens presented during infection.

The second challenging issue for an EBOV vaccine is to have the ability to protect against infection from aerosolized viruses. This has been a long-time concern, as aerosols are the most likely form for these viruses as a biological threat (4), and it has been shown that aerosolizing EBOV is an efficient way to cause infection in animal models (12, 18). Our studies showed that EBO7 completely protected macaques against aerosolized ZEBOV infection by 28 days after vaccination with only a single dose and could protect against aerosol challenge by the more lethal challenge with aerosolized SEBOV with a two-dose vaccination regime. To the best of our knowledge, this is the first such demonstration of protection of cynomolgus macaques against aerosol challenge with SEBOV.

In addition, we found that aerosolized SEBOV appears to be an even more significant respiratory pathogen than aerosolized ZEBOV and can cause necrotic pneumonia and pleuritis, in addition to the typical severe lesions in multiple vital organs. Immunochemical staining of lung lesions showed large amounts of viral antigen, confirming that viral replication indeed caused the pneumonia. The severity shown by these findings is in sharp contrast to that seen in macaques challenged with ZEBOV by the aerosol route, where only on histopathologic examination were there indications of viral involvement in the lungs (18). With ZEBOV, the lung lesions are typically a mild to moderate interstitial pneumonia that appears to play a minor role in mortality in comparison with the widespread lesions found in other organs, such as the liver and spleen. Furthermore, histological analysis revealed foci of fibrous pleural adhesions in two of the vaccinated macaques, indicating an initial infection of the lungs by SEBOV, even in the presence of robust vaccine-induced virus-specific antibody responses similar to those seen in NHPs completely protected against parenteral challenges by ZEBOV and SEBOV or aerosol challenge by ZEBOV. However, our results show that vaccination with EBO7 vaccine is able to restrict the initial SEBOV infection in the lungs and protect the macaques from deadly systematic infection or symptoms of the disease.

Regardless of the differences in lung pathology induced by the two types of EBOV, our studies have shown that EBOVs can efficiently cause systematic infection through the respiratory route of exposure and, therefore, that it is critically important for a vaccine to induce protection against aerosolized EBOVs. In response to our findings, we are currently examining the mucosal route of vaccine delivery to determine if it can induce an added level of immune protection in the lungs to prevent the pulmonary sequelae seen in our vaccinated macaques. Nevertheless, we did make the important finding that protection against airway infection of the more lethal SEBOV could be achieved with two-dose administration of EBO7, which in part could be due to the induction of significantly higher SEBOV-specific antibody titers.

A third issue for vaccines based on viral vectors is the possibility that preexisting immunity to the vaccine vector may hamper their efficacy. The CAdVax vaccine described here is based on Ad5, a common and benign human respiratory virus against which a significant percentage of the human population acquires immunity during childhood. Consequently, much of the adult population is seropositive for neutralizing antibodies

to Ad5 (1, 26). It is commonly perceived that these Ad5 neutralizing antibodies would neutralize any Ad5-based vaccines and limit their effectiveness. This concern has been heightened by experiments in mice showing that Ad5 vectors lose their effectiveness after repeated administration of high levels of Ad5 virus (20). In these and other similar studies, Ad5-based vaccines were administered at the peak of the initial immune response against Ad5, and in this context, alternative serotypes of Ad perform better by avoiding the acute phase of immunity against Ad5. However, such studies do not precisely replicate the character of preexisting immunity in humans, where the initial response has waned and memory immunity has been maintained. Moreover, previous studies in NHPs have suggested that preexisting immunity to Ad5 can be overcome by increasing the vaccine dose (5).

To investigate the potential impact of preexisting immunity toward the Ad5 vector, we analyzed immune responses after repeatedly exposing NHPs to wild-type Ad5 virus. The results of these studies support the concept that the high antibody level achieved during an initial acute immune response is transient and declines to a lower-level memory immunity within weeks. In the natural situation, the memory immunity will be able to mount a secondary immune response to prevent subsequent infection. However, we conducted a stringent examination of memory immunity by repeated injection of the NHPs with high doses of Ad5 virus. Again, the acute immune responses subsided to a lower level of sustained immune response, although it appeared to increase after each injection. Our hypothesis is that the lower level of the memory immune response can neutralize only a fraction of the vaccine at the site of injection and, therefore, a vaccine dose that is sufficient to compensate for the amount of the vaccine neutralized will be effective even in the presence of preexisting immunity. Indeed, our results have shown that the medium dose (10^9 PFU) used in our study generated antibody responses in Ad5-immune animals equivalent to those seen in Ad5-naïve animals, suggesting that preexisting immunity can be overcome with moderate increases in the vaccine dose. Even at a low dose of vaccine (10^8 PFU), a second dose boosted the antibody response to levels similar to those of the higher doses, and these NHPs were completely protected against lethal aerosol challenge with ZEBOV. This is consistent with the interpretation that preexisting antibodies can neutralize only a portion of the vaccine dose and that the uptake of Ad vector into target cells (in minutes) is much more rapid than the development of secondary immune responses (in days), allowing more than adequate levels of vaccine vector to enter cells, express the GP antigens, and induce immunity. Considering the importance of this issue to the development of vectored vaccines, we believe additional studies with a large number of NHPs, especially using wild-type Ad5, to examine the impact of preexisting vector immunity on vaccine efficacy are justifiable.

While the combination of the two CAdVax-based vectors for Ebola virus (EBO7) and Marburg virus (M8) GPs constitute the first effective multivalent filovirus vaccine (33), a mixture of vesicular stomatitis virus (VSV)-EBOV chimeric viruses has recently been demonstrated to provide protection against multiple filoviruses (14). These attenuated chimeric VSV-EBOV viruses were constructed by insertion of the filovirus glycoprotein gene into the VSV background, thus requiring a separate chimeric virus to express each glycoprotein antigen (19). In contrast, the CAdVax platform can express multiple antigens and in addition, is nonreplicating, and the underlying adenovirus vector has proven safe in numerous clinical trials.

In summary, the need for EBOV vaccines is clear, from both public health and biodefense standpoints. The most effective EBOV vaccines will be those that are safe and capable of providing protection against multiple species of filovirus. Our studies have shown that developing single-vector vaccines against multiple virus strains is feasible using the CAdVax platform. Additionally, our data have demonstrated for the first time the feasibility of a single vaccine to protect NHPs against aerosol infections by both ZEBOV and SEBOV.

ACKNOWLEDGMENTS

We thank Carlton Rice for animal care and the staff of the Center for Aerobiological Sciences, USAMRIID, particularly Mathew Lackemeyer, for assistance with aerosol exposures. We also thank Ana Kuehne, Jay Wells, and Ramon Ortiz for their technical assistance.

Work on filoviruses at USAMRIID was funded by the Defense Threat Reduction Agency (project number 1.1C0001_07_RD_B and Cooperative Agreement DAMD17-02-2-0035) and in part by U.S. Public Health Service grant AI070382 from the National Institute of Allergy and Infectious Diseases to J.Y.D.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

REFERENCES

1. **Appaiahgari, M. B., R. M. Pandey, and S. Vrati.** 2007. Seroprevalence of neutralizing antibodies to adenovirus type 5 among children in India: implications for recombinant adenovirus-based vaccines. Clin. Vaccine Immunol. **14:**1053–1055.
2. **Bermejo, M., J. D. Rodriguez-Teijeiro, G. Illera, A. Barroso, C. Vila, and P. D. Walsh.** 2006. Ebola outbreak killed 5000 gorillas. Science **314:**1564.
3. **Borio, L., T. Inglesby, C. J. Peters, A. L. Schmaljohn, J. M. Hughes, P. B. Jahrling, T. Ksiazek, K. M. Johnson, A. Meyerhoff, T. O'Toole, M. S. Ascher, J. Bartlett, J. G. Breman, E. M. Eitzen, Jr., M. Hamburg, J. Hauer, D. A. Henderson, R. T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G. J. Nabel, M. T. Osterholm, T. M. Perl, P. Russell, and K. Tonat.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA **287:**2391–2405.
4. **Bray, M.** 2003. Defense against filoviruses used as biological weapons. Antiviral Res. **57:**53–60.
5. **Casimiro, D. R., L. Chen, T. M. Fu, R. K. Evans, M. J. Caulfield, M. E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D. C. Freed, K. A. Wilson, S. Dubey, D. M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J. G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G. J. Heidecker, H. C. Perry, N. Persaud, T. J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A. J. Bett, D. B. Volkin, E. A. Emini, and J. W. Shiver.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J. Virol. **77:**6305–6313.
6. **Centers for Disease Control and Prevention.** 2009. Known cases and outbreaks of Ebola hemorrhagic fever, in chronological order. Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia. http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/ebola/ebolatable.htm.
7. **Centers for Disease Control and Prevention.** 2009. Known cases and outbreaks of Marburg hemorrhagic fever, in chronological order. Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Georgia. http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/marburg/marburgtable.htm.
8. **Centers for Disease Control and Prevention.** 2005. Outbreak of Marburg virus hemorrhagic fever—Angola, October 1, 2004-March 29, 2005. MMWR Morb. Mortal. Wkly. Rep. **54:**308–309.
9. **Clontech Laboratories.** 2007. Adeno-X Expression System 1 user manual, p. 44-45. Clontech Laboratories, Mountain View, CA.
10. **Feldmann, H., T. W. Geisbert, P. B. Jahrling, H. D. Klenk, S. V. Netesov, C. J. Peters, A. Sanchez, R. Swanapoel, and V. E. Volchkov.** 2005. Filoviridae, p. 645–653. *In* C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus taxonomy: VIIIth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego, CA.

11. **Feldmann, H., S. M. Jones, K. M. Daddario-DiCaprio, J. B. Geisbert, U. Stroher, A. Grolla, M. Bray, E. A. Fritz, L. Fernando, F. Feldmann, L. E. Hensley, and T. W. Geisbert.** 2007. Effective post-exposure treatment of Ebola infection. PLoS Pathog. **3:**e2.
12. **Geisbert, T. W., K. M. Daddario-Dicaprio, J. B. Geisbert, D. S. Reed, F. Feldmann, A. Grolla, U. Stroher, E. A. Fritz, L. E. Hensley, S. M. Jones, and H. Feldmann.** 2008. Vesicular stomatitis virus-based vaccines protect nonhuman primates against aerosol challenge with Ebola and Marburg viruses. Vaccine **26:**6894–6900.
13. **Geisbert, T. W., K. M. Daddario-DiCaprio, K. J. Williams, J. B. Geisbert, A. Leung, F. Feldmann, L. E. Hensley, H. Feldmann, and S. M. Jones.** 2008. Recombinant vesicular stomatitis virus vector mediates postexposure protection against Sudan Ebola hemorrhagic fever in nonhuman primates. J. Virol. **82:**5664–5668.
14. **Geisbert, T. W., J. B. Geisbert, A. Leung, K. M. Daddario-DiCaprio, L. E. Hensley, A. Grolla, and H. Feldmann.** 2009. Single-injection vaccine protects nonhuman primates against infection with Marburg virus and three species of Ebola virus. J. Virol. **83:**7296–7304.
15. **Geisbert, T. W., L. E. Hensley, T. Larsen, H. A. Young, D. S. Reed, J. B. Geisbert, D. P. Scott, E. Kagan, P. B. Jahrling, and K. J. Davis.** 2003. Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. Am. J. Pathol. **163:**2347–2370.
16. **Groseth, A., H. Feldmann, and J. E. Strong.** 2007. The ecology of Ebola virus. Trends Microbiol. **15:**408–416.
17. **Hevey, M., D. Negley, P. Pushko, J. Smith, and A. Schmaljohn.** 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology **251:**28–37.
18. **Johnson, E., N. Jaax, J. White, and P. Jahrling.** 1995. Lethal experimental infections of rhesus monkeys by aerosolized Ebola virus. Int. J. Exp. Pathol. **76:**227–236.
19. **Jones, S. M., H. Feldmann, U. Stroher, J. B. Geisbert, L. Fernando, A. Grolla, H. D. Klenk, N. J. Sullivan, V. E. Volchkov, E. A. Fritz, K. M. Daddario, L. E. Hensley, P. B. Jahrling, and T. W. Geisbert.** 2005. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat. Med. **11:**786–790.
20. **Kobinger, G. P., H. Feldmann, Y. Zhi, G. Schumer, G. Gao, F. Feldmann, S. Jones, and J. M. Wilson.** 2006. Chimpanzee adenovirus vaccine protects against Zaire Ebola virus. Virology **346:**394–401.
21. **Leffel, E. K., and D. S. Reed.** 2004. Marburg and Ebola viruses as aerosol threats. Biosecur. Bioterror. **2:**186–191.
22. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
23. **Mellquist-Riemenschneider, J. L., A. R. Garrison, J. B. Geisbert, K. U. Saikh, K. D. Heidebrink, P. B. Jahrling, R. G. Ulrich, and C. S. Schmaljohn.** 2003. Comparison of the protective efficacy of DNA and baculovirus-derived protein vaccines for EBOLA virus in guinea pigs. Virus Res. **92:**187–193.
24. **Moe, J. B., R. D. Lambert, and H. W. Lupton.** 1981. Plaque assay for Ebola virus. J. Clin. Microbiol. **13:**791–793.

24a. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.

25. Reference deleted.
26. **Nwanegbo, E., E. Vardas, W. Gao, H. Whittle, H. Sun, D. Rowe, P. D. Robbins, and A. Gambotto.** 2004. Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. Clin. Diagn. Lab. Immunol. **11:**351–357.
27. **Raviprakash, K., D. Wang, D. Ewing, D. H. Holman, K. Block, J. Woraratanadharm, L. Chen, C. Hayes, J. Y. Dong, and K. Porter.** 2008. A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. J. Virol. **82:**6927–6934.
28. **Rubinchik, S., J. S. Norris, and J. Y. Dong.** 2002. Construction, purification and characterization of adenovirus vectors expressing apoptosis-inducing transgenes. Methods Enzymol. **346:**529–547.
29. **Rubinchik, S., D. Wang, H. Yu, F. Fan, M. Luo, J. S. Norris, and J. Y. Dong.** 2001. A complex adenovirus vector that delivers FASL-GFP with combined prostate-specific and tetracycline-regulated expression. Mol. Ther. **4:**416–426.
30. **Rubinchik, S., J. Woraratanadharm, J. Schepp, and J. Y. Dong.** 2003. Improving the transcriptional regulation of genes delivered by adenovirus vectors. Methods Mol. Med. **76:**167–199.
31. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. U. S. A. **93:**3602–3607.
32. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681–684.
33. **Swenson, D. L., D. Wang, M. Luo, K. L. Warfield, J. Woraratanadharm, D. H. Holman, J. Y. Dong, and W. D. Pratt.** 2008. Vaccine to confer to nonhuman primates complete protection against multistrain Ebola and Marburg virus infections. Clin. Vaccine Immunol. **15:**460–467.
34. **Towner, J. S., T. K. Sealy, M. L. Khristova, C. G. Albarino, S. Conlan, S. A. Reeder, P. L. Quan, W. I. Lipkin, R. Downing, J. W. Tappero, S. Okware, J. Lutwama, B. Bakamutumaho, J. Kayiwa, J. A. Comer, P. E. Rollin, T. G. Ksiazek, and S. T. Nichol.** 2008. Newly discovered Ebola virus associated with hemorrhagic fever outbreak in Uganda. PLoS Pathog. **4:**e1000212.
35. **Volchkov, V. E., S. Becker, V. A. Volchkova, V. A. Ternovoj, A. N. Kotov, S. V. Netesov, and H. D. Klenk.** 1995. GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology **214:**421–430.
36. **Walsh, P. D., T. Breuer, C. Sanz, D. Morgan, and D. Doran-Sheehy.** 2007. Potential for Ebola transmission between gorilla and chimpanzee social groups. Am. Nat. **169:**684–689.
37. **Wang, D., M. Hevey, L. Y. Juompan, C. M. Trubey, N. U. Raja, S. B. Deitz, J. Woraratanadharm, M. Luo, H. Yu, B. M. Swain, K. M. Moore, and J. Y. Dong.** 2006. Complex adenovirus-vectored vaccine protects guinea pigs from three strains of Marburg virus challenges. Virology **353:**324–332.
38. **Wang, D., N. U. Raja, C. M. Trubey, L. Y. Juompan, M. Luo, J. Woraratanadharm, S. B. Deitz, H. Yu, B. M. Swain, K. M. Moore, W. D. Pratt, M. K. Hart, and J. Y. Dong.** 2006. Development of a cAdVax-based bivalent Ebola vaccine that induces immune responses against both the Sudan and Zaire species of Ebola virus. J. Virol. **80:**2738–2746.
39. **Wang, D., A. L. Schmaljohn, N. U. Raja, C. M. Trubey, L. Y. Juompan, M. Luo, S. B. Deitz, H. Yu, J. Woraratanadharm, D. H. Holman, K. M. Moore, B. M. Swain, W. D. Pratt, and J. Y. Dong.** 2006. De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses. Vaccine **24:**2975–2986.
40. **Warfield, K. L., C. M. Bosio, B. C. Welcher, E. M. Deal, M. Mohamadzadeh, A. Schmaljohn, M. J. Aman, and S. Bavari.** 2003. Ebola virus-like particles protect from lethal Ebola virus infection. Proc. Natl. Acad. Sci. U. S. A. **100:**15889–15894.
41. **Warfield, K. L., D. L. Swenson, G. G. Olinger, W. V. Kalina, M. J. Aman, and S. Bavari.** 2007. Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. J. Infect. Dis. **196**(Suppl. 2)**:**S430–S437.
42. **World Health Organization.** 7 December 2007. Ebola haemorrhagic fever in Uganda—update. Global Alert and Response, World Health Organization, Geneva, Switzerland. http://www.who.int/csr/don/2007_12_07/en/index.html.
43. **World Health Organization.** 2005. Outbreak news: Marburg haemorrhagic fever, Angola—update. Wkly. Epidemiol. Rec. **80:**297.
44. **World Health Organization.** 2007. Outbreak news: Ebola virus haemorrhagic fever, Democratic Republic of the Congo—update. Wkly. Epidemiol. Rec. **82:**345.
45. **World Health Organization.** 2007. Outbreak news: Marburg haemorrhagic fever, Uganda. Wkly. Epidemiol. Rec. **82:**297.